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Insight in the PCB-degrading functional community in long-term contaminated soil under bioremediation

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Abstract
Purpose A small-scale bioremediation assay was developed in order to get insight into the functioning of a polychlorinated biphenyl (PCB) degrading community during the time course of bioremediation treatment of a contaminated soil. The study was conducted with the aim to better understand the key mechanisms involved in PCB-removal from soils.
Materials and methods Two bioremediation strategies were applied in the assay: (a) biostimulation (addition of carvone as inducer of biphenyl pathway, soya lecithin for improving PCB bioavailability, and xylose as supplemental carbon source) and (b) bioaugmentation with selected seed cultures TSZ7 or *Rhodococcus* sp. Z6 originating from the transformer station soil and showing substantial PCB-degrading activity. Functional PCB-degrading community was investigated by using molecular-based approaches (sequencing, qPCR) targeting *bphA* and *bphC* genes, coding key enzymes of the upper biphenyl pathway, in soil DNA extracts. In addition, kinetics of PCBs removal during the bioremediation treatment was determined using gas chromatography mass spectrometry analyses.
Results and discussion *bphA*-based phylogeny revealed that bioremediation affected the structure of the PCB-degrading community in soils, with *Rhodococcus*-like bacterial populations developing as dominant members. Tracking of this population further indicated that applied bioremediation treatments led to its enrichment within the PCB-degrading community. The abundance of the PCB-degrading community, estimated by quantifying the copy number of *bphA* and *bphC* genes, revealed that it represented up to 0.3% of the total bacterial community. All bioremediation treatments were shown to enhance PCB reduction in soils, with approximately 40% of total PCBs being removed during a 1-year period. The faster PCB reduction achieved in bioaugmented soils suggested an important role of the seed cultures in bioremediation processes.
Conclusions The PCBs degrading community was modified in response to bioremediation treatments, leading to partial removal of PCBs from contaminated soil. The abundance of the PCB-degrading community was increased, and *Rhodococcus*-like bacterial population was identified as its key player. Combination of molecular approaches with chemical analyses is of prime interest to monitor microbial processes involved in the bioremediation treatment. They offer new insight in the managing of PCBs contaminated sites providing the opportunity to design the bioremediation strategy based on on-site evaluation of PCBs degrading ability of the soil microbial community.

Keywords Bioremediation · *bph* gene · PCB-degrading community · Polychlorinated biphenyls · *Rhodococcus*

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1 Introduction

Polychlorinated biphenyls (PCBs) are man-made chemicals that have been widely used for different industrial and commercial purposes before recognition of their harmful impacts on the environment (Faroon et al. 2000). Even though their production has been banned more than 30 years ago, PCBs still represent ecological and health issues worldwide because of their persistence, toxicity, and potential accumulation in the food web. In spite of toxicological effects of PCBs, various microbial strains have been isolated and characterized that are able to catabolize PCBs, either through anaerobic or aerobic processes (Borja et al. 2005; Field and Sierra-Alvarez 2008). Their use in bioremediation is considered as a promising strategy for cleaning-up PCB-contaminated environments.

The well-characterized biphenyl degradation pathway is ubiquitous in PCB-degrading bacteria (Pieper 2005). BphA, BphB, BphC, and BphD enzymes, comprising the biphenyl upper pathway, catalyze the conversion of biphenyl to benzoate and 2-hydroxypenta-2,4-dienote as final metabolites. Dioxygenases BphA (encoded by the *bphA* gene) and BphC (encoded by the *bphC* gene) are considered as key enzymes, catalyzing critical reactions in PCB-transformation. By incorporating two hydroxyl groups into the biphenyl ring, BphA increases PCBs reactivity and renders them more susceptible to enzymatic ring fission reactions catalyzed by BphC (Bruhlmann and Chen 1999). It has been suggested that PCBs carrying chlorine substituents at 2,3 (5,6) positions of the biphenyl ring are converted through dioxygenase attack on the 3,4 (4,5) positions. At present, only a few bacterial strains have been shown to possess capacities for such PCB transformation (Gibson et al. 1993; Komancova et al. 2003).

Despite extensive laboratory studies to gain better insight into the microbial degradation of PCBs and to overcome the limiting factors, efficient degradation is still difficult to achieve, and a decline of PCB-degrading efficiency from shake culture to soil microcosm experiments is observed. One of the main limiting factors is the need for an inducer to promote PCBs co-metabolism. Although biphenyl has been shown to be the best promoter of PCB degradation, its use in remediation is hindered by its own toxicity and low solubility (Luo et al. 2007). Several natural compounds, such as terpenes, have been shown to induce PCB co-metabolism (Gilbert and Crowley 1998; Singer et al. 2000; Tandlich et al. 2001). The use of surfactants, which could increase the availability of hydrophobic PCBs to microorganisms, has also been proposed for enhancing PCB degradation (Fava et al. 2003). Stimulating the growth and PCB-degrading activity of bacteria within the indigenous microbiota (biostimulation) and enriching contaminated soil with active PCB degraders (bioaugmentation) have been proposed as strategies to enhance PCB degradation in soil. However, the implementation of these bioremediation strategies is made difficult by the complex interactions occurring between microbiota and soil ecosystem components (Iwamoto and Nasu 2001; Ohtsubo et al. 2004). The development of reliable and efficient bioremediation methods will require a better understanding of the biological factors involved in the soil PCB-degradation processes. The availability of culture-independent molecular methods based on direct soil DNA extraction and analyses using polymerase chain reaction (PCR) approaches allows targeting and identification of the PCB-degrading bacterial population in contaminated environments. Studying bacteria in PCB-contaminated soil, Leigh et al. (2007) have revealed discrepancies between culture-dependent and independent methods, the latter allowing the identification of new genera of bacteria (*Pseudonocardia*, *Kribella*, *Nocardiodes*, and *Sphingomonas*)—potentially able to degrade PCBs. Several authors have assessed the genetic potential of the PCB-degrading bacterial population in contaminated soils and sediments by developing PCR assays to target the *bphA* or *bphC* genes (Erb and Wagnerdobler 1993; Layton et al. 1994; Hoostal et al. 2002). However, only a few studies have been dedicated to changes in the microbial community and more specifically to the PCB-degrading bacterial population during bioremediation of PCB-contaminated soil (Luepromchai et al. 2002; Singer et al. 2003; Di Toro et al. 2006). These experiments suggested a positive impact of the addition of different active PCB degraders, a complex consortium of microorganisms, earthworms, and plants, on the abundance of biphenyl and/or monochlorobenzoate-degrading bacterial populations and the levels of the *bphA* and *bphC* genes.

The aim of our work was to study the impact of bioremediation treatments on the genetic diversity, abundance, and catabolic activity of the PCB-degrading functional community in soil contaminated with PCBs. A small-scale bioremediation assay, involving both biostimulation and bioaugmentation strategies, was designed by using transformer station soil that has been contaminated with PCBs in 1991. Mixed culture TSZ7 and its metabolically active member, *Rhodococcus* sp. Z6, previously enriched from the same transformer station soil, expressing substantial PCB-degrading activity, were used as seed cultures (Petric et al. 2007). Based on previous studies with TSZ7 and Z6 cultures, carried out in liquid media and soil microcosms (Hrsak and Petric 2005), carvone, xylose, and soya lecithin were selected as amendments for enhancing PCB degradation. The genetic diversity of the PCB-degrading community in soil was studied by cloning and sequencing the *bphA* gene. The abundance of *bphA* and *bphC* genes was determined by quantitative PCR assays. Specific primers designed for this

study allowed the differentiation of two groups of *bphC* sequences corresponding to gram-positive and gram-negative PCB-degrading bacteria. Despite the fact that the qPCR method is often used to estimate the abundance of targeted microbial groups or catabolic genes, to our knowledge, apart from our paper, the paper recently published by Correa et al. (2009) is the only one in which this method has been used to estimate the relative abundance of *bph* genes correlated with PCB degradation

2 Materials and methods

2.1 Contaminated soil

PCB-contaminated soil (approximately 500 kg) was excavated from a 10×4 m plot on the location of the transformer station (TS 110/35 kV) damaged during warfare operation in 1991 in Zadar area (Croatia). Prior to starting the bioremediation assay, the soil was sieved (1-cm mesh), to remove stones and coarse plant material. Then the soil sample was manually homogenized by repeated sieving and mixing. The soil was characterized as a typical Mediterranean "terra rossa," characterized by a predominance of clay and silt particles [51.4% clay, 30.6% silt, 18.0% sand, organic matter 39.0 $g kg^{-1}$, organic carbon 22.6 $g kg^{-1}$, total nitrogen 2.1 $g kg^{-1}$, C/N yield 11, pH 7.2 (in water)].

2.2 Seed cultures

A mixed bacterial culture TSZ7, and its metabolically active member *Rhodococcus* sp. Z6, was used as seed cultures for the bioremediation assay. Culture TSZ7 previously enriched from the transformer station soil was efficient in degrading di- to hexa-chlorinated PCBs (Petric et al. 2007). TSZ7 culture constituted of eight culturable members among which the Z6 strain was shown to be the key player in PCB-degrading activity of this TSZ7 culture. The seed culture inoculum was grown at 30°C under agitation in 5 l Erlenmeyer flasks containing 1 l of phosphate-buffered mineral salts PAS medium supplemented with biphenyl (5 $g l^{-1}$) as the sole carbon source (Bedard et al. 1986).

2.3 Small-scale bioremediation assay

Three plastic containers (0.84×0.41×0.16 m) coated with thick aluminum foil were filled with the sieved and homogenized contaminated soil (approximately 90 kg). Each container comprised two compartments representing replicates of each bioremediation treatment. Each container was equipped with an irrigation system run by a peristaltic pump (Minipuls 2, Gilson Medical Electronics, Inc., WI, USA; Fig. 1). The containers were maintained under semi-controlled conditions (an open room protected from rain). The soil physico-chemical parameters were regularly measured: moisture content and temperature using an HH2 moisture meter equipped with WET sensor (Delta-T Devices Ltd., Cambridge, UK) and pH using InoLab pH 720 equipped with SenTix 81 pH sensor (WTW GmbH, Weilheim, Germany). The bioremediation assay consisted of: (a) a biostimulation treatment with PAS medium containing xylose as supplemental carbon source (1 $g l^{-1}$), carvone as inducer of the PCB-degrading pathway (100 $mg l^{-1}$), and soya lecithin as surfactant for enhancing PCBs bioavailability (5 $g l^{-1}$; BS treatment) and (b) two bioaugmentation treatments, one employing repeated inoculation with mixed culture TSZ7 (BAM treatment), and the other with the Z6 strain (BAP treatment). The cultures (500 ml) suspended in PAS medium (1.5 l) containing the same organic amendments as in the biostimulation treatment were inoculated to the soil (approximately 1×10^{12} $cfu kg^{-1}$ of soil). Treatments were repeated at 2-week intervals to maintain soil moisture and cell density and to ensure an adequate supply of amendments.

Prior to starting the assay, the initial PCB concentration of the contaminated soil in three containers was determined by gas chromatography–mass spectrometry (GC–MS). Approximately 500 g of soil was collected from each compartment of each container (n=6). Each sample was mixed and two sub-samples were further analyzed (n_{tot}= 12). By comparison with the Aroclor 1248 standard

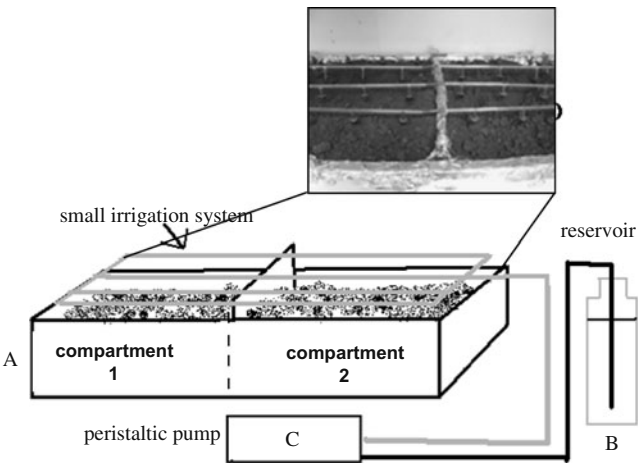

Fig. 1 Scheme of the small-scale bioremediation assay: three containers (0.84×0.41×0.16 m) comprised of **a** two separated compartments filled with PCB-contaminated soil (collected in the proximity of the damaged transformer station, Zadar, Croatia) equipped with **b**, **c** irrigation systems were kept in an open room protected from rain. Soil moisture, temperature, and pH were monitored throughout the assay. One container was set up to test biostimulation treatment (addition of carvone, soya lecithin, and xylose) and two others to test bioaugmentation treatments (addition of mixed culture TSZ7 or *Rhodococcus* sp. Z6 as seed cultures apart from carvone, soya lecithin, and xylose)

(Supelco, Bellefonte, USA), PCB concentration was estimated to 31.4±4 μg PCB per gram of soil. GC–MS analysis revealed the presence of 41 di- to hepta-chlorinated PCBs in concentrations ranging from 0.001 to 6.249 $\mu g g^{-1}$ of soil with tri- and tetra-chlorinated PCBs as the main soil contaminants (0.4 to 6.2 $\mu g g^{-1}$; Table 1). No significant differences (Kruskal–Wallis, $p<0.05$) were found between PCB concentrations in sub-samples thereby confirming the homogeneity of the contamination in the soil.

For chemical analyses, a composite soil sample composed of nine randomly selected soil samples (approximately 500 g) was collected from each compartment of three treatments (BS, BAM, and BAP) throughout the assay ($n=6$). In addition, at each sampling time, initial contaminated soil preserved at +4°C was sub-sampled ($n=1$) and used as a control for chemical analyses. Soil samples (BS, BAM, BAP, and control) were ground in a mortar and sieved (2 mm mesh), mixed and subsampled into two replicates for further GC–MS analyses ($n_{tot}=14$).

For the molecular analyses, at the end of the 18-month assay, three soil samples were collected from the six compartments of the different bioremediation treatments (BS, BAM, and BAP; $n=18$). Eighteen months after the beginning of the assay, three soil samples (0–10 cm) were collected at the transformer station site and used as non-treated soil (NT, $n=3$). Soil samples were kept at −20°C until their use ($n_{tot}=21$).

2.4 Isolation and characterization of biphenyl-degrading bacteria

Potential PCB-degrading bacteria were tracked by plating decimal dilutions of soil suspensions (1 g equivalent dry weight homogenized in 10 ml of 1M physiological solution) onto the surface of PAS agar plates. After incubation at 30°C (7–10 days) under biphenyl atmosphere bacteria exhibiting 2,3-dihydroxybiphenyl 1,2-dioxygenase activity (colonies surrounded by a yellow zone after spraying with an aqueous solution of 2,3-dihydroxybiphenyl) were counted. Biphenyl-degrading isolates were purified by repeated streaking on PAS agar plates supplemented with biphenyl vapor. Total DNA was extracted from isolates using the "QIAGEN Blood & Cell Culture DNA Midi Kit" (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. The isolates were identified by sequencing 16S rRNA amplicons which had been amplified with 27_f and 1492_r universal primers (Gurtler and Stanisich 1996). The obtained sequences were deposited in the GenBank NCBI database under accession numbers GQ899188–GQ899198 and compared with known 16S rRNA sequences.

2.5 DNA extraction from soil

Total DNA was extracted from 1.5-g soil samples following the procedure described by Martin-Laurent et al. (2001) and proposed for ISO standardization (ISO/DIS11063). The method consists of three major steps: (a) mechanical and chemical lyses of the cells in the extraction buffer using a cell disrupter (B. Braun Biotech International, Germany), (b) protein precipitation with sodium acetate, and (c) nucleic acids precipitation (cold isopropanol) and washing (70% ethanol).

The obtained soil DNAs were purified using polyvinyl polypyrrolidone and Sepharose 4B spin columns (Sigma-Aldrich, USA) and the purification kit "Geneclean Turbo Kit" (Qbiogen, France) according to the manufacturer's instructions. After checking for soil DNA integrity by electrophoresis on 1% agarose gel, the DNA was

Table 1 Chemical identity and mass fraction of dominant PCB congeners representing approx. 70% of PCB-contamination in soil[a]

GC-MS peak assignment	PCB IUPAC nomenclature	PCB chlorine substitution	PCB mass fraction in soil ($\mu g\ g^{-1}$)	
1	18, 17	2,2′,4; 2,2′,5	0.40	± 0.055
2	32, 16	2,4′,6; 2,2′,3	0.86	± 0.108
3	28, 31	2,4,4′; 2,4′,5	6.23	± 0.749
4	52	2,2′,5,5′	1.49	± 0.175
5	49	2,2′,4,5′	1.37	± 0.175
6	48, 47	2,2′,4,5; 2,2′,4,4′	1.10	± 0.155
7	44	2,2′,3,5′	1.40	± 0.171
8	42	2,2′,3,4′	0.91	± 0.139
9	71, 41, 64	2,3′,4′,6; 2,2′,3,4; 2,3,4′,6	2.17	± 0.296
10	40	2,2′,3,3′	0.39	± 0.076
11	74	2,4,4′,5	1.46	± 0.209
12	70	2,3′,4′,5	1.75	± 0.228
13	66, 95	2,3′,4,4′; 2,2′,3,5′,6	2.71	± 0.371
14	60	2,3,4,4′	0.92	± 0.150

Quantitative and qualitative analyses of PCBs were performed by gas chromatography–mass spectrometry in comparison with Aroclor 1248 used as a standard

[a] Soil was collected in the vicinity of the damaged transformer station, Zadar, Croatia

quantified at 260 nm using a BioPhotometer (Eppendorf, Germany). Soil DNA was likewise extracted from the control NT soil.

2.6 qPCR assays

The abundance of the PCB-degrading community was estimated by performing qPCR assays targeting *bph* sequences in soil DNA extracts (BS, BAM, BAP, and NT soil samples) with an ABI 7900 HT Fast Real-Time PCR System (Applied Biosystems, USA). Reactions were performed in a 20-µl final reaction volume containing 10 µl SYBR green PCR Master Mix (Absolute QPCR SYBR Green Rox ABgene, France), 1 µM of each primer, 100 ng of T4 gene 32 (QBiogene, France), and 20 ng of the soil DNA used as a template. The *bphA* sequences were amplified using a previously described primer set 463_f and 674_r targeting the gene coding for a dioxygenase catalyzing the first step of PCB transformation (Demnerova et al. 2005). Cycling conditions for the amplification of *bphA* were as follows: 15 min at 95°C; ten cycles of 15 s at 95°C, 30 s at 60°C with a touchdown −0.5°C by cycle, 30 s at 72°C, 30 s at 82°C; 35 cycles of 15 s at 95°C, 30 s at 55°C, 30 s at 72°C, 30 s at 82°C followed by a dissociation step. Specific primer sets were designed to target two groups of *bphC* sequences, gram-positive *Rhodococcus*-like sequences [*bphC*(Rh)] and gram-negative *Pseudomonas*-like sequences [*bphC*(Ps)] coding for a dioxygenase catalyzing opening of the PCB biphenyl ring (Table 2). Primers were designed from multiple alignment of *bphC* sequences retrieved from the GenBank database (list of sequences is given in Table S1 provided as Electronic supplementary material). Specificity of the newly developed primers was validated by cloning and sequencing the amplified PCR products, after which optimized qPCR protocols were developed for each of the targeted sequences. The *bphC* amplification conditions were as follows: 15 min at 95°C; 35 cycles of 15 s at 95°C, 30 s at 60°C, 30 s at 72°C, and 30 s at 80°C followed by a dissociation step. The abundance of the global bacterial community was estimated by 16S rRNA qPCR assays using the 341_f–534_r primer set as described previously (Henry et al. 2004). One non-template control was run for each qPCR. Calibration of qPCR was carried out with a serial dilution (from 10^1 to 10^6 copies) prepared with the appropriate cloned target sequence. The standard calibration curves relating the log of the copy number of the target sequence as a function of the cycle threshold (C_t) were as follows: $\log(bphA) = -0.26 \times C_t + 9.259$ ($r^2 = 0.995$), $\log[bphC(\mathrm{Rh})] = -0.29 \times C_t + 10.725$ ($r^2 = 0.999$), $\log[bphC(\mathrm{Ps})] = -0.28 \times C_t + 11.258$ ($r^2 = 0.999$) and $\log(16\mathrm{S\ rRNA}) = -0.30 \times C_t + 11.8$ ($r^2 = 0.997$).

2.7 *bphA* sequencing and phylogenetic analysis

For phylogenetic studies, *bphA* amplicons were cloned into the plasmid vector pGEM-T easy according to the manufacturer's instructions (Promega, France) to establish one library for each treatment (four in total). Approximately 40 clones were picked from four different libraries (approximately ten for each library) and checked for the correct insert size by vector targeted PCR. *bphA* nucleotide sequences were determined using the GenomeLab DTCS Quick Start kit (Beckman Coulter, USA) with T7 primer in a Ceq8000 Sequencer, according to the manufacturer's instructions. Sequences were deposited in the GenBank database under the accession numbers GQ411216–GQ411249. The *bphA* sequences were compared with known nucleotide sequences using the Blast program (http://blast.ncbi.nlm.nih.gov/Blast.cgi). A phylogenetic tree was constructed from multiple alignments carried out with the ClustalX program and the data obtained were analyzed with NJ Plot software using the neighbor-joining method. The accession numbers of the strains used to construct the tree were as follows: EF596933 (*Paenibacillus* sp. B257), FJ416870.1 (*Cuprivaidus* sp), EU812171.1 (*Achromobacter* sp. BP3), CP000272.1 (*Burkholdera xenovorans* LB400), AJ251217.1 (*Pseudomonas* sp. B4), AY504981.1 (*Pseudomonas aeruginosa* JI104), D32142.1 (*Rhodococcus* sp. RHA1), CP000432.1 (*Rhodococcus jostii* RHA1), AP011117.1 (*Rhodococcus opacus* B4), EF527236.1 (*Rhodococcus* sp. L4), DQ813271.1 (*Rhodococcus aetherivorans I24) and U24277.1 (Rhodococcus erythopolis), and HM153082 (Rhodococcus sp. Z6).*

Table 2 Primers used for amplifying targeted *bphA* and *bphC* sequences from soil DNA extracts

Gene	Primer	Nucleotide sequence (5′→3′)	PCR fragment size (bp)	Annealing T (°C)	Reference
bphA	463_f 674_r	CGC GTS GMV ACC TAC AAR G GGT ACA TGT CRC TGC AGA AYT GC	211	55	Demnerova et al. (2005)
bphC	$\mathrm{bphC(Rh)}_f$ $\mathrm{bphC(Rh)}_r$	GAT GAC GAT GCT TCG CTG CGG CAA GCT GTG GTG RCG CKS GTT	153	60	This study
bphC	$\mathrm{bphC(Ps)}_f$ $\mathrm{bphC(Ps)}_r$	ACT TCC TGC ACT GCA AYG GAC TTG TGR CCC CAC ATG	317	60	This study

2.8 PCB extraction and quantification

PCBs were extracted from 10-g soil sub-samples following the procedure described by Peris et al. (2000). Briefly, the first step included five repetitions of solvent extraction (15 ml, ϕ(*n*-hexane/acetone)=1:1) using an ultrasonic bath. The extracts were combined, reduced to 10 ml under a stream of nitrogen and cleaned by adding 0.5 g of Cu and 0.5 g of Hg. The organic layer was dried in a nitrogen flow. After dissolving the dry residues in *n*-hexane (1 ml) and adding sulfuric acid (1 ml), the samples were centrifuged (1,000×*g*, 2–3 min) and the organic supernatant was submitted to GC–MS analysis using Varian Saturn II GC–MS system (Varian, Walnut Creek, CA, USA) equipped with an ion trap detector, under conditions previously described (Petric et al. 2007). The PCB content in the soil ($\mu g g^{-1}$ dry weight) was estimated by comparison with the Aroclor 1248 standard. Reduction of PCBs during bioremediation treatments, expressed as a percentage, was calculated by comparing PCB mass fraction in bioremediated soil with that determined in the control soil sample and was expressed as mean value (± standard deviation).

2.9 Statistical analyses

To determine significant differences in *bphA*/16SrRNA and *bphC*/16SrRNA ratios between different bioremediation treatments, the Kruskal–Wallis statistical test was performed using XLSTAT.

3 Results

3.1 Abundance of cultivable biphenyl-degrading bacterial populations

Biphenyl-degrading bacterial populations expressing 2,3-dihydroxybiphenyl 1,2-dioxygenase activity (BphC), i.e., potential PCB-degrading population, were regularly counted throughout the assay. In the BAM and BAP soils, colonies expressing BphC activity were observed from the first month whereas in the BS soil they were detected 6 months after the beginning of the bioremediation assay. The BphC bacterial population ranged from 3.8 to 15×10^6 $cfu g^{-1}$ in BAM soil, from 1.8 to 9.6×10^6 $cfu g^{-1}$ in BAP soil, and from 2.6 to 4.3×10^6 $cfu g^{-1}$ in BS soil throughout the assay with no significant differences observed no matter the bioremediation treatment considered. Despite our efforts, only ten bacterial isolates expressing BphC activity, were successfully further purified. The 16S rRNA sequences of these isolates shared high similarity (95–99%) with that of *Rhodococcus* species.

3.2 Phylogenetic diversity of *bphA* sequences in soils

The phylogenetic diversity of the PCB-degrading functional community was estimated by targeting the *bphA* gene coding for the first enzyme involved in PCB transformation. Four libraries were prepared from BS, BAP, BAM, and NT soils. Comparison of the obtained *bphA* sequences with known sequences recorded in the GenBank database revealed that those amplified from bioremediated soils (BS, BAP, and BAM) exhibited 97–99% similarity to the aromatic ring hydroxylating dioxygenases genes (biphenyl, isopropylbenzene, and benzene) of different bacterial strains belonging to the genus *Rhodococcus* (Fig. 2). On the contrary, the *bphA* sequences amplified from the NT soil produced nearest matches (89–98%) to *bphA* sequences of gram-negative bacteria belonging to *Pseudomonas*, *Burkholderia*, *Achromobacter*, and *Cupravidus* genera.

3.3 Abundance of the *bph* sequences in soils

The abundance of the PCB-degrading functional community was estimated by amplifying the *bphA* and *bphC* sequences by qPCR. The specific richness of the functional communities in the soils was assessed by comparing the relative abundances of targeted communities with those of the global bacterial community. Values were calculated as *bph* sequence copy numbers per copy number of 16S rRNA sequences (%). PCR amplifications were done using DNA extracted directly from the soil as a template. As is evident from Fig. 3, panel A, *bphA* sequences were obtained from both treated (BS, BAP, and BAM) and control soil (NT). However, no *bphC* amplicons were obtained from NT soil, while in all bioremediated soils, gram-positive *Rhodococcus*-like *bphC* sequences were amplified with group-specific primers. No gram-negative *Pseudomonas*-like *bphC* sequences could be amplified from the DNA extracts. The relative abundance of *bphA* sequences in the soils ranged from 0.02–0.14% (largest difference observed between BAM and NT soil, Kruskal–Wallis, $p<0.05$) while the relative abundances of *bphC* sequences ranged from 0.08% to 0.31% (no significant differences observed between three bioremediated soils). The efficiencies of the PCR assays were: 86% for *bphA* and 95% and 93% for *Rhodococcus*-like and *Pseudomonas*-like *bphC*, respectively.

3.4 PCB reduction in soils

Continuous monitoring of physico-chemical parameters showed that bioremediation treatments ensured soil moisture between 40% and 50%, while the temperature varied depending on the outdoor conditions (autumn, winter from 12°C to 18°C spring, summer from 18°C to 28°C) and pH values ranged from 6.8 to 7.05.

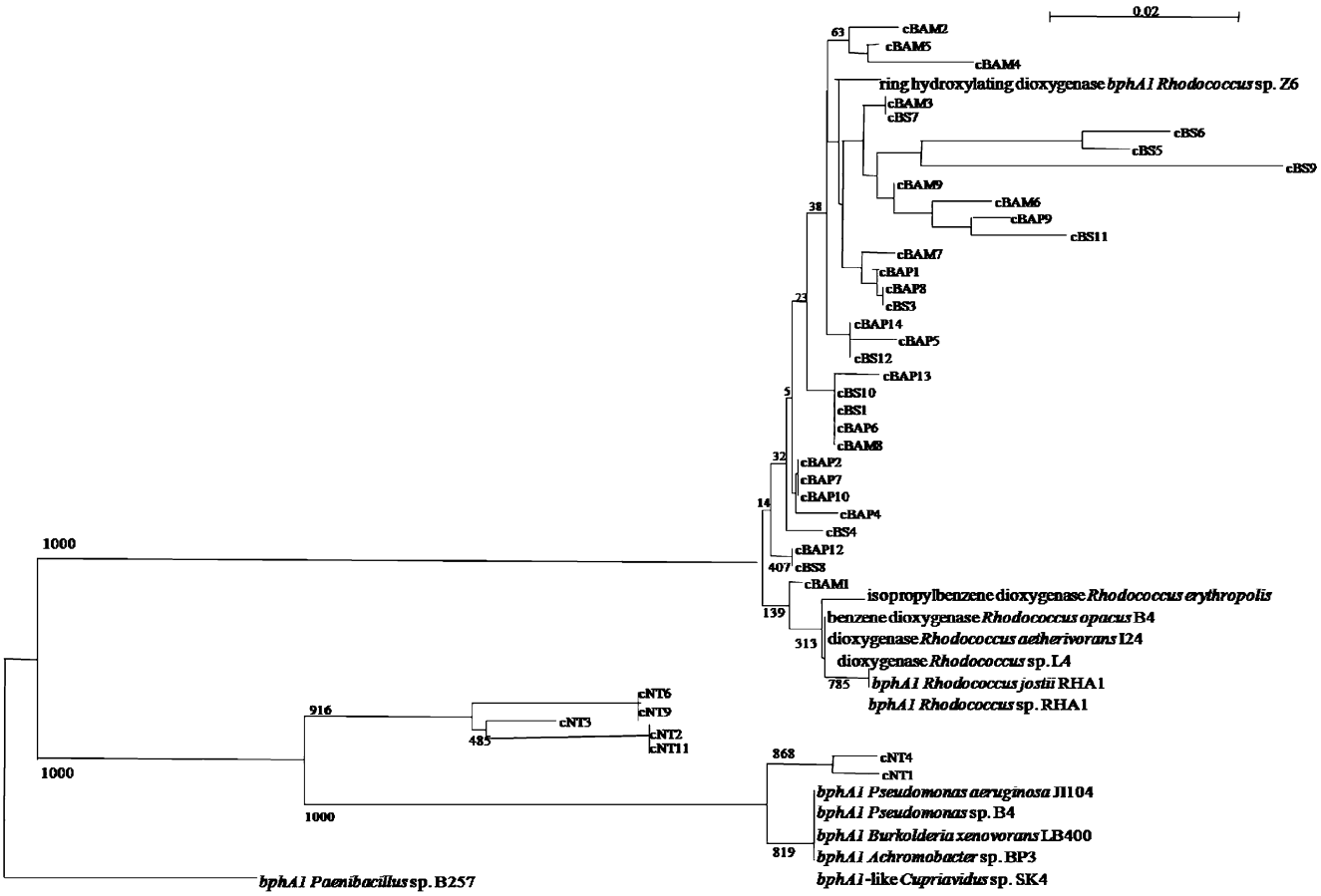

Fig. 2 Phylogenetic relationship between *bphA* sequences (partial, ~200 bp) amplified from BAM, BAP, BS, and NT soil and closely related sequences retrieved from the GenBank database. Phylogenetic tree was constructed using the neighbor-joining method with *bphA* sequences of *Paenibacillus* sp. B257 used as an out-group. *cBAM* clone sequences amplified from BAM soil (xylose, carvone, soya lecithin, and TSZ7 mixed culture amendments), *cBAP* clone sequences amplified from BAP soil (xylose, carvone, soya lecithin, and *Rhodococcus* sp. Z6 amendments), *cBS* clone sequences amplified from BS soil (xylose, carvone, and soya lecithin amendments), *cNT* clone sequences amplified from the control soil

GC–MS analyses of the soil extracts revealed that, by the end of the bioremediation treatments approximately 40% of total PCBs had been removed from the contaminated soil (see Fig. 3, panel B). While for the BAM treatment PCB dissipation kinetics starts immediately, 2 and 3 months lag phases were observed for BAP and BS treatments, respectively. Maximum percentage of PCB reduction was achieved after 9, 11, and 13 months in BAP, BAM, and BS soils, respectively. Thereafter, no further PCB reduction was observed. Percentage reduction of individual PCB congeners, comprising 73% of total PCB contamination in soil (21 tri- and tetra-chlorobiphenyls), are presented in Fig. 3, panel C. Similar patterns in reduction of the selected PCB congeners were observed in BAM, BAP, and BS soils. In general, higher overall PCB reduction was observed for trichlorobiphenyls (tri-CBs; 50–70% reduction) than for tetra-CBs (30–40% reduction). It is noteworthy that the trichlorobiphenyls PCB28 and PCB31, which account for almost 20% of soil PCB contaminations, were substantially removed (67% reduction). Furthermore, a spectrum of PCB congeners with chlorine atoms on different positions of the biphenyl ring (di-ortho, di-para, or di-meta substitution), such as 2,2′-diCB, 2,6-diCB, 4,4′-diCB, 2,2′,5,5′-tetraCB, 2,2′,4,4′-tetraCB, 2,4,4′,5-tetraCB, 2,3,4,4′-tetraCB, and 2,2′,3,3′-tetraCB, were efficiently reduced.

4 Discussion

Previous enrichment of the PCB-degrading mixed culture TSZ7 from transformer station soil (TS) damaged during the war in Croatia and contaminated with approximately 32 μg $PCB\,g^{-1}$ has suggested the presence of indigenous PCB-degrading functional community and has indicated a potential for natural attenuation (Petric et al. 2007). This has been further confirmed by the isolation of *Rhodococcus* sp. Z6 from the culture TSZ7 able to degrade a wide range of di- to penta-PCBs. Although the contaminated soil has been shown to harbor a PCB-degrading bacterial community, the amount of PCBs remaining at the TS site suggests that

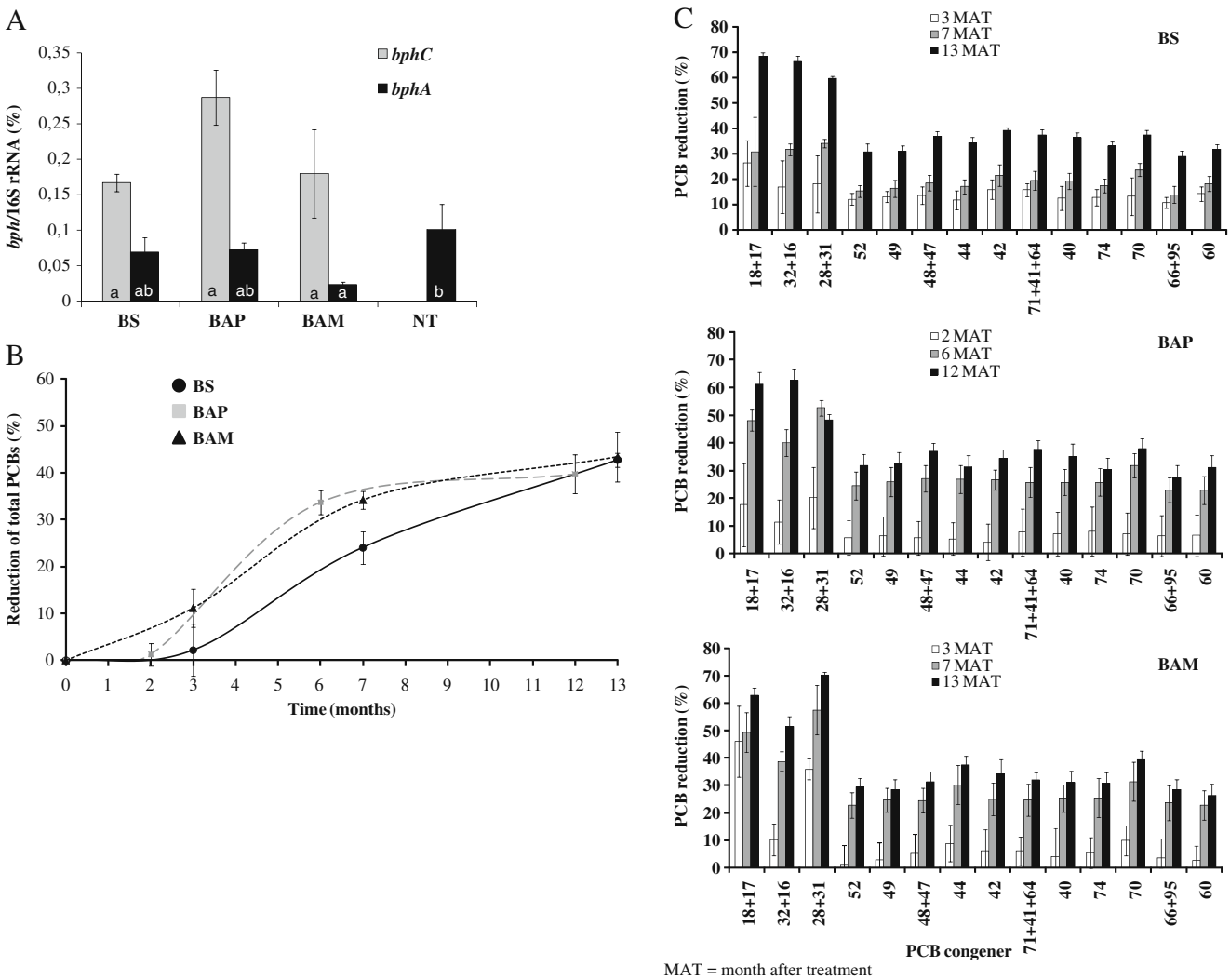

Fig. 3 **a** Relative abundances (mean±SD, n=6 for each treatment) of *bphA* and *bphC* sequences in soils under biostimulation treatment (BS, xylose, carvone, and soya lecithin amendments), bioaugmentation treatments with *Rhodococcus* sp. Z6 (BAP, xylose, carvone, soya lecithin, and *Rhodococcus* sp. Z6 culture amendments) or mixed culture TSZ7 (BAM, xylose, carvone, soya lecithin, and mixed culture TSZ7 amendments) and in control soil (NT, n=3). Sequences found in BS, BAP, and BAM soils were *Rhodococcus*-like while those in NT soil were *Pseudomonas*-like. Significantly different values between different soils for the same gene sequence are marked with *lowercase letters* (Kruskal–Wallis test, $p<0.05$). **b** Reduction of total PCB mass fractions (mean±SD, n=4 for each treatment) in BS, BAP, and BAM soils. PCB reduction in soils was determined by gas chromatography–mass spectrometry analyses by comparing mass fractions in bioremediated soils with that determined in the control soil. **c** Reduction of individual PCB congeners (mean±SD, n=4 for each treatment) representing approximately 70% of PCB contamination in BAM, BAP, and BS soils during the bioremediation treatments. Depletion of PCBs was determined by gas chromatography–mass spectrometry analyses by comparing mass fractions in bioremediated soils with that determined in the control soil

natural attenuation is poor. With the aim to clean up this site, we set up a small-scale bioremediation assays to assess the functioning of the PCB-degrading bacterial community focusing primarily on their phylogenetic diversity, abundance, and catabolic activity. To accelerate PCB-degradation processes non-toxic natural compounds, shown to promote PCB-transformation of the TSZ7 culture and strain Z6, were used: carvone, as the inducer of the PCB-degrading pathway, xylose as the supplemental carbon source to promote soil microbiota growth, and soya lecithin, to enhance PCBs availability (Hrsak and Petric 2005).

Culturable biphenyl-degrading bacteria were detected in all treated soils. These potential PCB degraders, harboring active 2,3-dihydroxybiphenyl enzyme (BphC), were identified as members of the genus *Rhodococcus*. Although culturable approach does not allow differentiation between bioaugmented and indigenous *Rhodococcus* populations, the observation of colonies with phenotype similar to *Rhodococcus* sp. strain Z6 (Petric et al. 2007) in BAM- and BAP-treated soil suggested that it was surviving after its inoculation in contaminated soil all along the assay. Due to the poor cultivability of soil microbes and to avoid the

biases associated with cultivable approaches, we applied molecular methods based on direct soil DNA extraction and further analyses based on polymerase chain reaction. The genetic diversity of the PCB-degrading bacterial community was estimated by amplifying, cloning, and sequencing the *bphA* sequences, coding the first enzyme involved in PCB transformation. The sequences amplified from bioremediated soil (BAM, BAP, and BS) could be clearly distinguished from those of the control soil (NT) by phylogenetic analysis. The *bphA* sequences amplified from BAM, BAP, and BS soils showed low phylogenetic diversity and clustered in a single phylogenetic group formed by several aromatic dioxygenases related to those of the bacteria belonging to the genus *Rhodococcus*. The similarity of sequences to biphenyl dioxygenase of the seed strain *Rhodococcus* sp. Z6 would indicate either (a) survival of the inoculants in the soil and/or (b) the development of bacterial populations analogous to that obtained by enrichment. The analyzed sequences showed similarity not only to biphenyl-dioxygenase (*bphA*) but also to isopropylbenzene (*ipbA*) and benzene (*bnzA*) dioxygenases. Interestingly, the catabolic pathways of different aromatic compounds are known to have overlapping specificities (Heald and Jenkins 1996; Baldwin et al. 2000). The observed dioxygenase diversity therefore suggests that, in the soil environment, PCB transformation may rely on a complex metabolic network of mosaic pathways cooperating in PCB oxidation. In contrast, the *bphA* sequences amplified in the control soil clustered into a phylogenetic group formed by biphenyl-dioxygenase sequences related to gram-negative bacteria. Even though the presence of *Rhodococcus*-like sequences in the bioaugmented soils could be expected, it was apparent from the biostimulation treatment that the addition of carvone and soya lecithin led to a switch in the genetic potential of the functional bacterial community from gram-negative to gram-positive bacterial populations, favoring development of the indigenous *Rhodococcus*-like PCB-degrading bacterial populations. In addition, the recovery of biphenyl-degrading *Rhodococcus* isolates, in accordance with this observation, suggests that such bacteria might play a key role in degrading PCBs in the soils under investigation. These results further support the idea that bacteria belonging to the genus *Rhodococcus*, which are known for their good survival in harsh environments, range of diverse catabolic genes, multiple degrading pathways, and robust physiology, are promising candidates for the bioremediation of PCB-contaminated sites (Larkin et al. 2005).

The abundance of the PCB-degrading bacterial community developing in soil under the bioremediation treatments was determined by targeting *bphA* and *bphC* sequences in qPCR assays. The results suggested that the PCB-degrading community harbored both genes coding for key enzymes of PCB transformation. Estimation of the relative abundances of the *bph* sequences to the 16S rRNA sequences in the total bacterial community revealed that the PCB-degrading community represented up to 0.3% of the total bacterial community. In addition, although the *bphA* primer set allowed amplification of the *bphA* sequences in a wide range of gram-positive and gram-negative PCB-degraders, separate primer sets were designed to either amplify gram-positive *Rhodococcus*-like or gram-negative *Pseudomonas*-like *bphC* sequences. As a result, we observed that bioremediation (both biostimulation and bioaugmentation) led to an increased abundance of the *Rhodococcus*-like *bphC* sequences in soil, whereas, on the contrary, *Pseudomonas*-like sequences were detected neither in bioremediated nor in non-treated control soil.

Interestingly, sequences of the *bphA* gene were also detected in the non-treated control soil and, compared to bioremediated soils, in greater abundance. This could lead to the erroneous conclusion that the bioremediation treatments did not have an effect on the *bphA* in soil. However, the phylogenetic studies showed that the *bphA* sequences in bioremediated and control soils clustered into two distinct groups, clearly indicating that bioremediation increased the abundance of the gram-positive like *bphA* sequence. Furthermore, the detection of *bph* gram-negative-like sequences in the control soil is in accordance with the fact that soil bacterial populations are known to possess *bph* genes that are responsible for degradation of various naturally occurring aromatics (Koh et al. 2000; Furukawa et al. 2004). However, it should be emphasized that *bph* genes expression is dependent on inducers and, in their absence, genes expression is poor. This could at least partly explain the poor PCB-degrading activity of indigenous bacteria observed at the TS-contaminated site. It was also apparent that the *bphC* sequences were detected at slightly higher levels than the *bphA* sequences in bioremediated soils. This could be due to the limitation of qPCR assays resulting from the fact that the efficiency of the *bphA* assay was slightly lower than that of the *bphC* assays. Alternatively, it can be hypothesized that the discrepancy was caused by the presence of several copies of the *bphC* sequences or could imply that the PCB-degrading community possesses alternative genes coding for the first step in PCB transformation.

PCB-contaminated soil collected at the transformer station site was shown to contain predominantly tri- to tetra-chlorinated and *ortho*-substituted congeners, thereby suggesting that higher chlorinated congeners could have been transformed to lower chlorinated congeners by the indigenous soil microbiota at the TS site. As previously suggested by several authors, the anaerobic dechlorination of higher-chlorinated PCBs is occurring in natural environments, but primarily in river sediments. Since previous experiments have shown that the cultures TSZ7 and Z6 are

efficient in the aerobic degradation of a wide range of lower-chlorinated congeners (Petric et al. 2007), they were expected to be efficient seed cultures for cleaning up the contaminated site under study.

GC–MS analysis of the PCB mass fractions in soils revealed that after 1-year of biostimulation and bioaugmentation PCB contamination has been reduced to approximately 12 $\mu g PCB\, g^{-1}$ of soil. This significant PCB removal is of particular interest since soils with a long history of PCB contamination, such as the TS soil, are known for their low PCBs bioavailability. Bioaugmentation performed with the mixed culture TSZ7 or *Rhodococcus* sp. Z6 resulted in faster PCB reduction than in soil subjected to biostimulation, suggesting a complementary role of the seed cultures in accelerating bioremediation processes. It is hypothesized that the lag phase observed in PCBs removal in BS soil did represent the time required for the indigenous PCB-degrading community to achieve critical abundance in response to biostimulation. Further confirmation of this hypothesis was the successful isolation of bacteria expressing BphC activity 6 months after starting of biostimulation.

Targeted tri- and tetra-chlorinated PCB congeners, which accounted for approximately 70% of the total PCB contamination, were efficiently reduced (up to 70%) from all treated soils, the tri-chlorinated congeners being reduced more extensively and rapidly. The observed reduction further suggests that the PCB-degrading bacterial community developing in bioremediated soils did exhibit a wide range of PCB-degrading capabilities, being able to transform structurally versatile congeners, even *ortho*-substituted ones, known for their high resistance to microbial degradation (Dai et al. 2002). Despite repeated biostimulation and bioaugmentation, PCB removal was only observed during the first year. It could be hypothesized that after an initial efficient phase of removal, the PCB concentrations dropped below the critical level required for their co-metabolic degradation. Furthermore, the accumulation of dead-end PCB metabolites such as chlorobenzoates might impair catabolic enzymes and limit further PCB transformation. This is supported by several studies reporting that a range of different metabolites produced during PCB transformation can act as bottlenecks in PCB degradation (Sondossi et al. 1992; Dai et al. 2002).

5 Conclusions

Our study, based on a small-scale bioremediation assay, was designed to describe functioning of PCBs degrading community during biostimulation and bioaugmentation treatments. It showed that over 1-year period, tested bioremediation treatments led to the removal of lower-chlorinated congeners accounting for approximately 70% of soil contamination. Combined use of molecular and chemical approaches allowed to demonstrate that both the abundance and composition of the PCB-degrading community were modified as a result of the bioremediation treatments and concomitantly PCB contamination was reduced. To our knowledge, this is the first report linking the functional diversity of the PCB-degrading communities with an observed PCB reduction due to bioremediation. Gram-positive *Rhodococcus*-like bacterial populations emerged as key members of the PCB-degrading community. The observed shift in composition and enrichment of the PCB degrading bacterial populations in response to the bioremediation treatments suggests that the use of molecular approaches to functionally characterize bacterial population with genetic potential to degrade PCBs could be of prime interest for monitoring and predicting the efficiency of bioremediation procedures for the removal of PCBs from contaminated soils. Further studies will aim at testing these approaches on a larger scale at the transformer station contaminated site.

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